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Friulimicins: Novel Lipopeptide Antibiotics with Peptidoglycan Synthesis Inhibiting Activity from *Actinoplanes friuliensis* sp. nov.

II. Isolation and Structural Characterization

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Four novel lipopeptide antibiotics, friulimicins A, B, C, and D, were isolated from cultures of *Actinoplanes friuliensis* HAG 010964 after fermentation in different nutrient media. The new compounds were separated by ion-exchange chromatography from the acidic lipopeptides of the amphomycin type also present in the culture fluid, compounds A-1437 A, B, E, and G. The principal constituent friulimicin B, $C_{59}H_{94}N_{14}O_{19}$, was structurally characterized by mass spectrometric investigations of its hydrolysis and partial degradation products and by sequencing of the cyclic acyl peptide. The NMR data of friulimycin B and the amphomycin constituent A-1437 B were completely assigned by a variety of 2-D experiments, and confirmed the structures determined by mass spectrometry. All 8 lipopeptides possess an identical peptide macrocycle as their central element, linked *via* a diaminobutyric acid *N*-terminal either to an acylated asparagine residue or, in the case of the amphomycin series, to an acylated aspartic acid residue. The structures of the amphomycins have now been revised to take account of the peptide framework described herein and the determined *cis*-configuration of the exocyclic double bond. As a consequence of their higher isoelectric points, the new compounds friulimicin A, B, C, and D have different properties than the amphomycins.

As a result of the spread of antibiotic resistance in antibacterial chemotherapy, novel agents are now urgently needed[1]. Infections caused by multiresistant pathogens that cannot be adequately treated with existing drugs are a particularly acute problem. Antibiotics that develop their antibacterial action by a novel mechanism, and are also well tolerated, are thus required.

An appropriate point of attack for therapeutically useful antibiotics is the synthesis of the bacterial cell wall. The acidic lipopeptide amphomycin[2,3] is a specific inhibitor of peptidoglycan synthesis[4,5], and inhibits phospho-MurNAc-pentapeptide transferase (EC 2.7.8.13), which is the enzyme that catalyses the first step of the lipid cycle during murein biosynthesis. As none of the antibiotics in current use have this step in cell wall synthesis as their site of action, amphomycin represents a highly promising starting point for the discovery of new treatment principles. Interestingly, these acidic lipopeptides are only inadequately described in the literature. Though glumamycin[6,7], crystallomycin[8], aspartocin[9,10], lasparto-mycin[11], tsushimycin[12,13], and other antibiotics belonging to the same group were isolated in addition to amphomycin, the mostly complex mixtures of antibiotics have not been adequately separated into individual compounds, nor their chemical structures have been unambiguously clarified. In a 1973 paper Bodanszky proposed a quasi-linear peptide structure[3] for the amphomycins, in which the aspartic acid *N*-terminal is acylated with (+)-3-isododecenoic acid or (+)-3-anteisotridecenoic acid. This was, however, inconsistent with the structures reported in 1965 by Fujino[7] for the identical glumamycin. It is therefore no surprise that the usefulness of acidic lipopeptide antibiotics in the treatment of bacterial infections has not yet been conclusively assessed.

In this paper we describe the isolation, from cultures of *Actinoplanes friuliensis* HAG 010964, of the new acidic lipopeptide antibiotics friulimicins A, B, C, and D and their characterization and structural elucidation. Accounts describing the strain *Actinoplanes friuliensis* HAG 010964 and the fermentation process that yields the new antibiotics have been published elsewhere[14,15].

Results

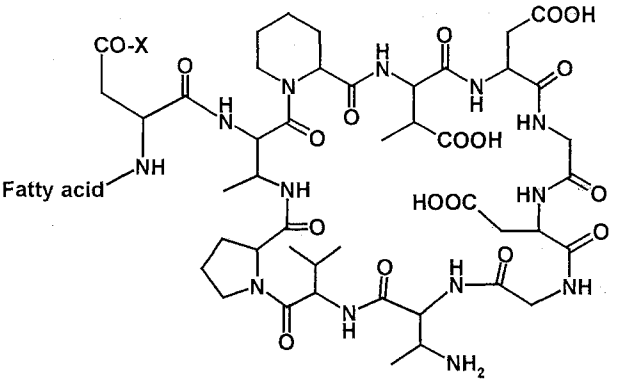
The starting materials for the compounds investigated are culture filtrates of *Actinoplanes friuliensis* HAG 010964, DSM 7358, which have been shown to contain substances with strong antibiotic activity, even against multiresistant strains[15].

Isolation

The friulimicins and the A-1437 antibiotics are highly water-soluble compounds present in the filtrate of the *Actinoplanes friuliensis* HAG 010964 culture fluid. The culture filtrates were loaded onto a column packed with Diaion HP20 adsorption resin, and the loaded resin was then washed with 10% 2-propanol in 10 mM phosphate buffer (pH 7.2). The antibiotics were then eluted with 50% 2-propanol in 10 mM phosphate buffer (pH 7.2). The column fractions containing the antibiotics were pooled, concentrated by ultrafiltration, and separated on a DEAE Sepharose anion-exchange column. Elution with a linear salt gradient in 50% methanol first gave the asparagine-containing antibiotics, in the order friulimicin A, C, B, and D, followed by the more strongly acidic Asp-type constituents A-1437 A, E, B, and G.

The enriched fractions were further purified by reversed-phase chromatography on MCI gel CHP20P or on LiChrospher RP Select B reversed-phase silica gel, eluting with a gradient of 10~30% acetonitrile in 20 mM sodium phosphate buffer (pH 7.2). The separated fractions were desalted on the same solid supports with 0~50% 2-propanol in water to give the pure antibiotics. Unsupplemented cultures gave mainly A-1437 B, whereas fermentations fed with valine afforded mainly friulimicin B in addition to A-1437 B. The 8 compounds described in this paper were present in all fermentations, though sometimes in very small amounts; the compounds friulimicins C and D and A-1437 E and H were obtained from the cultures grown on a media supplemented with isoleucine. Figure 1 shows the general structural formula of the isolated antibiotics, and Table 1 lists some of their physicochemical properties.

Fig. 1. Structures of the lipopeptide antibiotics from *Actinoplanes friuliensis* HAG 010964.



	X	Fatty acid
A-1437 A	OH	Δ3-isotridecenoic acid
A-1437 B	OH	Δ3-isotetradecenoic acid
Friulimicin A	NH_2	Δ3-isotridecenoic acid
Friulimicin B	NH_2	Δ3-isotetradecenoic acid
A-1437 E	OH	Δ3-anteisotridecenoic acid
Friulimicin C	NH_2	Δ3-anteisotridecenoic acid
A-1437 G	OH	Δ3-anteisopentadecenoic acid
Friulimicin D	NH_2	Δ3-anteisopentadecenoic acid

Physicochemical Properties of the Lipopeptides

The isolated antibiotics are colorless compounds with very high solubility in water and methanol. They are acidic lipopeptides whose amino acid analyses after hydrolysis with constantly boiling hydrochloric acid consistently yield the amino acids Asp (3), β-methyl-Asp (1), Gly (2), 2,3-diaminobutyric acid (Dab: 2), Pro (1), pipecolinic acid (Pip: 1), and Val (1). This is consistent with the amino acid composition previously reported in the literature for antibiotics of the amphomycin group[16]. The measured p*K*a values of friulimicin B—which is clearly less acidic than amphomycins A-1437 A, B, E, and G—were 8.5 (p*K*a1), 4.6 (p*K*a2), 4.1 (p*K*a3), and 3.1 (p*K*a4). The isoelectric point is 4.4, compared with values of between 3.5 and 3.6 reported for amphomycin. Dynamic light scattering measurements showed that in aqueous solution

Table 1. Physico-chemical properties of friulimicins A, B, C, and D and A-1437 A, B, E, and G.

Compound	A-1437 A	A-1437 B	Friulimicin A	Friulimicin B	A-1437 E	Friulimicin C	A-1437 G	Friulimicin D
Appearance[a]	Colourless amorphous solids							
Molecular formula	$C_{58}H_{91}N_{13}O_{20}$	$C_{59}H_{93}N_{13}O_{20}$	$C_{58}H_{92}N_{14}O_{19}$	$C_{59}H_{94}N_{14}O_{19}$	$C_{58}H_{91}N_{13}O_{20}$	$C_{58}H_{92}N_{14}O_{19}$	$C_{60}H_{95}N_{13}O_{20}$	$C_{60}H_{96}N_{14}O_{19}$
Molecular weight	1290.44	1304.46	1289.45	1303.48	1290.45	1289.45	1318.49	1317.52
ESI-MS (*m/z*), (M+H)$^+$	1290.8	1304.8	1289.9	1303.9	1290.8	1289.9	1318.8	1317,8
X =	-OH (Asp)	-OH (Asp)	-NH_2 (Asn)	-NH_2 (Asn)	-OH (Asp)	-NH_2 (Asn)	-OH (Asp)	-NH_2 (Asn)
Fatty acid	Δ3,*iso*-C_{13}	Δ3,*iso*-C_{14}	Δ3,*iso*-C_{13}	Δ3,*iso*-C_{14}	Δ3,*ai*-C_{13}	Δ3,*ai*-C_{13}	Δ3,*ai*-C_{15}	Δ3,*ai*-C_{15}
Amino acid composition [a]	Asp (3), Me-Asp (1), Gly (2), Pro (1), pipecolic acid (1), 2,3-diamino butyric acid (2), Val (1).							
UV λ_{max} nm (MeOH) [a]	End absorption							
IR ν_{max} cm^{-1} [a]	3400, 3060, 2960, 2920, 1660, 1590, 1530, 1450 and 1400 cm^{-1}							
Solubility of Na^+-salts [a] : soluble insoluble	Water (>300 mg / mL), methanol (>300 mg / mL), hexane.							
HPLC[b], retention time (minutes)	9.95	14.24	8.89	12.34	9.59	8.54	20.06	17.27

[a] in each case. [b] Column, LiChrospher 60RP-select B, 5μ, (4.0mm x 250mm), oven temperature, 40°C; Flow rate, 1 ml/min; UV detection at 210 nm; Solvent, CH_3CN : 0.1% H_3PO_4 = 44 : 56.

only about 2% of the antibiotic is present in monomeric form; for the other 98% a hydrodynamic particle radius of R_h=66 nm was calculated, corresponding to a micelle size of many millions of daltons.

Structure Elucidation

The mass spectrometric investigations described below were carried out for friulimicin B and A-1437 B. In an initial step, the peptides were subjected to acidic hydrolysis and subsequent derivatization (formation of methyl esters and trifluoroacetamides). The amino acids were identified by GC/MS. The identity of the unusual amino acids pipecolinic acid (Pip) and β-methylaspartic acid (MeAsp) was confirmed by high-resolution mass spectrometry, and α- and β-methylaspartic acid were distinguished by comparing MS/MS spectra.

Incomplete hydrolysis resulted in the identification of a dipeptide Val-Pro and a tripeptide Val-Pro-Dab. An unsaturated C_{14} fatty acid (FA, $C_{14}H_{26}O_2$) was also identified, lending further support to the assumption that the peptides belong to the class of lipopeptides. High-resolution FAB mass spectra gave a molecular mass M=1302.6884, corresponding to a formula $C_{59}H_{94}N_{14}O_{19}$, which is in agreement with the theoretical value of 1302.6820 calculated for the eleven amino acids and the fatty acid minus 12 molecules of water.

The complete structure of the lipopeptides was deduced by extensive high-resolution FAB measurements of the fragments and pseudomolecular ions. Some of the key fragments are summarized in Table 2. The smallest fragment of friulimicin B shown to contain the lipid residue is *m/z* 323. High-resolution mass spectrometry of this ion yielded the formula $C_{18}H_{31}N_2O_3$. Since the structure of the fatty acid moiety was known, we concluded that the fatty acid is linked to the *N*-terminus of an asparagine residue:

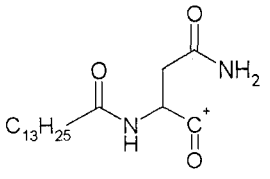
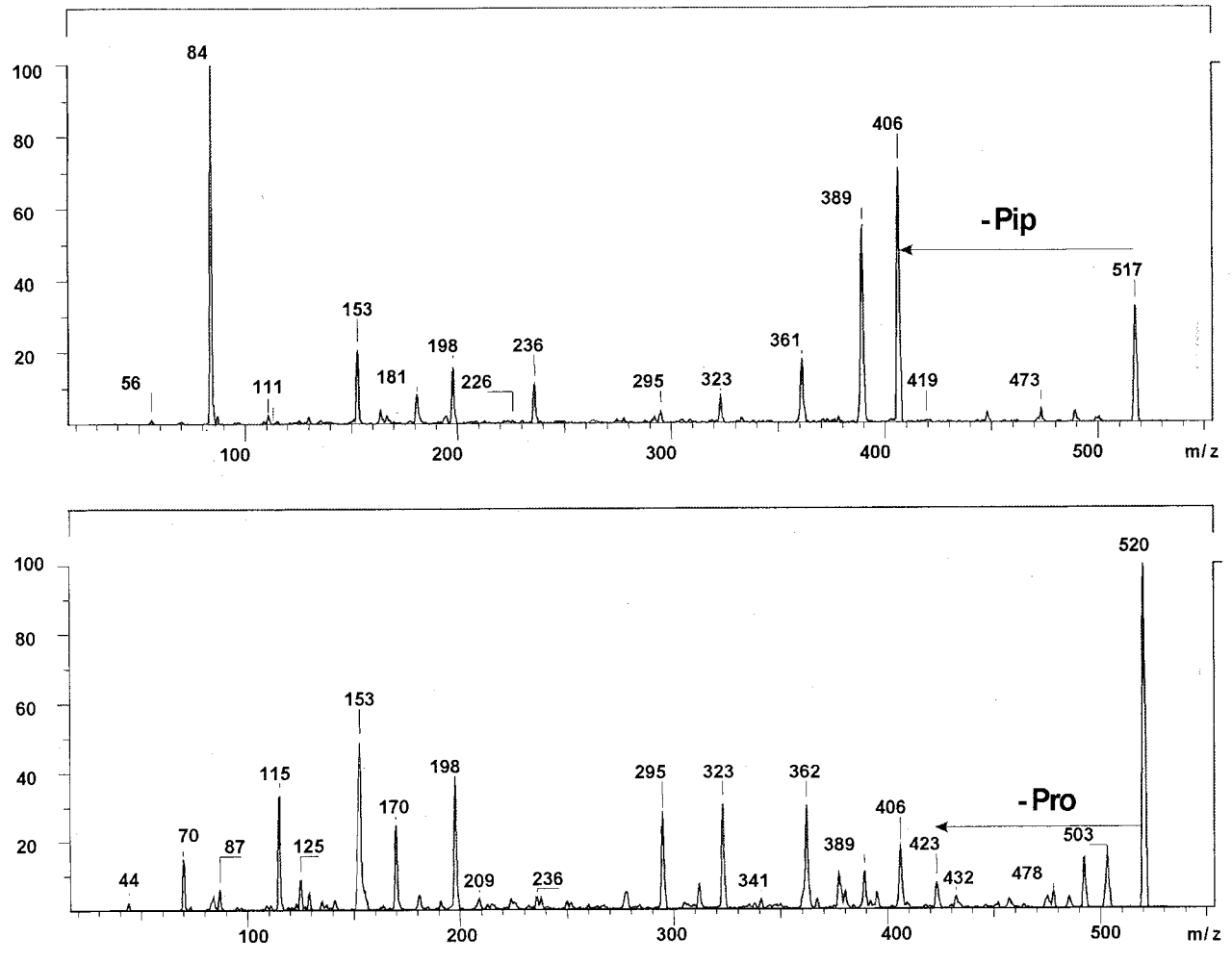


Table 2. High-resolution measurements of fragments and the pseudomolecular ion of friulimicin B under FAB conditions and corresponding elemental compositions

Measured mass*	Theoretical mass	Deviation (mmu)	Elemental formula
1303.6927	1303.6898	-2.2	$C_{59}H_{95}N_{14}O_{19}$
938.3965	938.4094	13.7	$C_{38}H_{58}N_{12}O_{16}$
920.3942	920.3988	5.0	$C_{38}H_{56}N_{12}O_{15}$
760.4586	760.4609	3.0	$C_{38}H_{62}N_7O_9$
631.4121	631.4183	9.8	$C_{33}H_{55}N_6O_6$
528.1864	528.1942	14.8	$C_{21}H_{30}N_5O_{11}$
520.3492	520.3499	1.3	$C_{27}H_{46}N_5O_5$
517.3355	517.3390	6.8	$C_{28}H_{45}N_4O_5$
406.2700	406.2706	1.5	$C_{22}H_{36}N_3O_4$
356.1469	356.1458	-3.1	$C_{15}H_{22}N_3O_7$
323.2353	323.2335	-5.6	$C_{18}H_{31}N_2O_3$

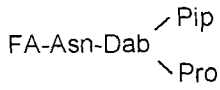
High-resolution measurements carried out by Dr. P. Carravatti (Spectrospin, Fällanden) with FT-ICR-MS on a Bruker CMX 47.

Fig. 2. MS/MS spectra of the FAB fragments *m/z* 517 and *m/z* 520.

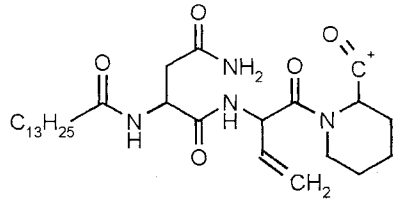


The molecular mass of the corresponding fragment in A-1437 B is increased by 1 Da, indicating the presence of an aspartic acid unit instead of asparagine.

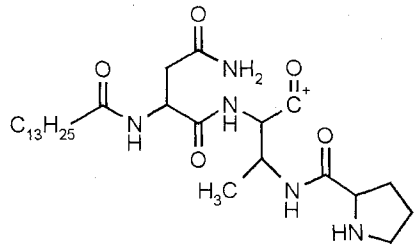
Further examination of the FAB mass spectra of friulimicin B reveals two key fragments containing FA and differing only by 3 Da, *m/z* 517 and *m/z* 520. The MS/MS spectra of these two ions reveal the presence of pipecolinic acid (for *m/z* 517) and proline (for *m/z* 520) respectively, as was deduced from the characteristic fragments *m/z* 84 and *m/z* 70 (Fig. 2). These findings can only be rationalized by a branching point connecting FA-Asn/Asp (*m/z* 323) and the fragments containing the pipecolinic acid and proline residues. The only amino acid in the molecule capable of serving as a branching point is diaminobutyric acid, which leads to an initial substructure containing FA, Asp[1]/Asn[1], Dab[2], Pip[3] and Pro[11]:



m/z 517: $C_{28}H_{45}N_4O_5$, corresponding to

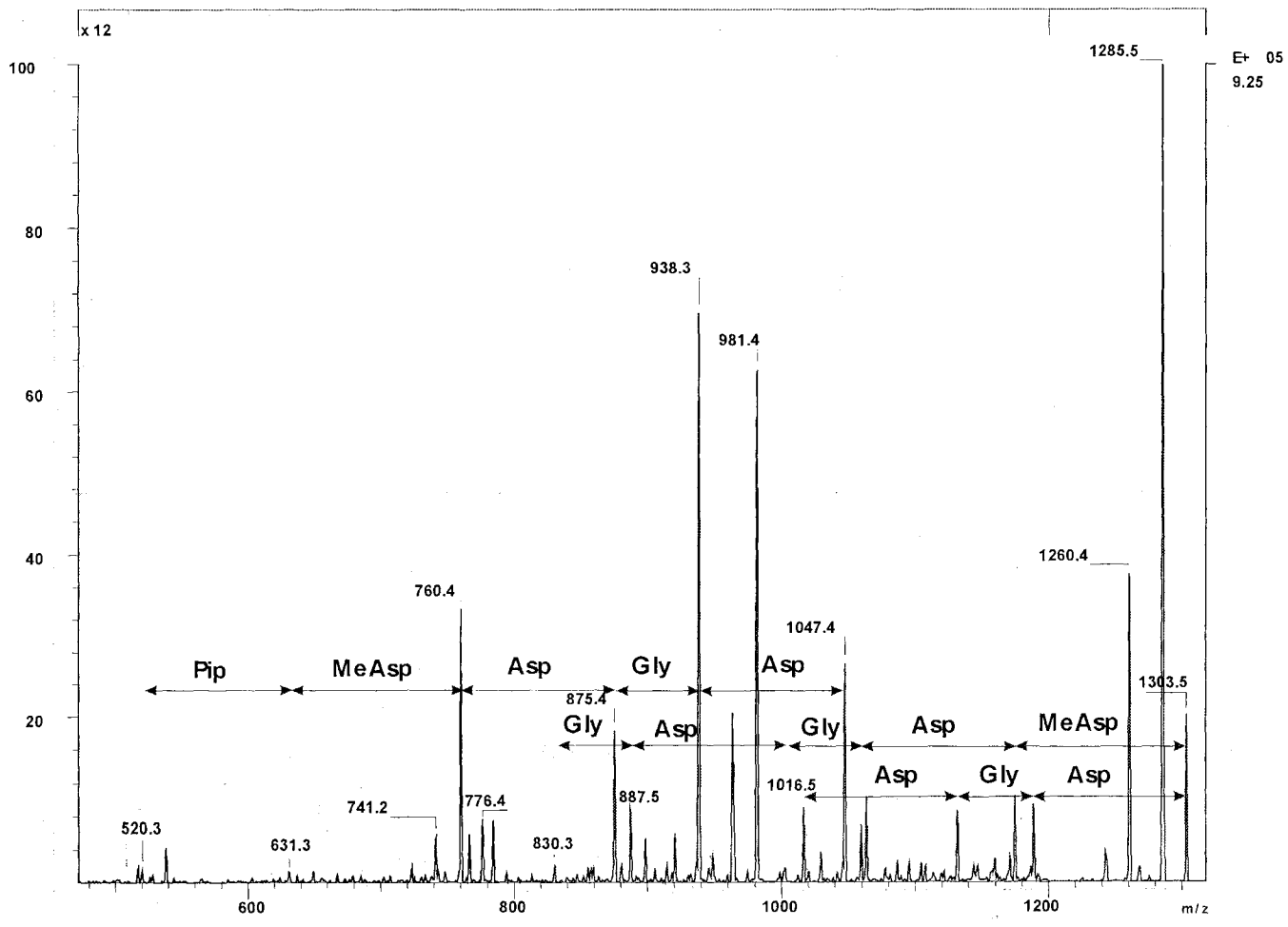


m/z 520: $C_{27}H_{46}N_5O_5$, corresponding to

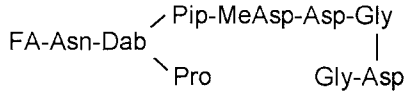


The presence of a branching point, together with the loss of 28 Da (CO) from the pseudomolecular ion and the failure to sequence the peptide by conventional methods, was strong evidence in support of a cyclic structure. From the MS/MS spectrum of the pseudomolecular ion, a second

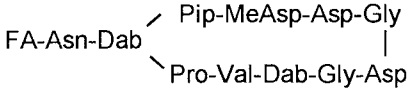
Fig. 3. MS/MS spectrum of the pseudomolecular ion *m/z* 1303 of friulimicin B.



substructure could be deduced as MH^+-[MeAsp^4-Asp^5-Gly^6-Asp^7-Gly^8] (Fig. 3). Amide bonds to proline residues are known to cleave very easily under MS/MS conditions[17]. With the assumption of a ring opening at this position, further fragments from *m/z* 520 to *m/z* 1047 (Fig. 3) could be assigned, pointing to a link between Pip^3 of substructure 1 and MeAsp^4 of substructure 2.



From the partial sequences described above and the di- and tripeptide identified from the hydrolysis product, the sequence including residues 1~8 and 10~11 was deduced and finally completed by the last amino acid Dab^9. The identification of the dipeptide Val-Pro allowed the sequence and retrosequence to be unequivocally distinguished, indicating proline as the *C*-terminus of the peptide and Asn as the *N*-terminus.



The fragmentation behavior, particularly for a series of non-FA-containing fragments (*e.g.* MH^+-[FA-Asn/Asp]: *m/z* 981), was the same for all the A-1437 peptides, indicating that the cyclic part must be identical for all the peptides investigated. Furthermore, amphomycin shows the same key fragments and fragmentation pattern, indicating a structure identical to that of A-1437 E. This is in agreement with one of the two structures originally proposed by M. FUJINO[7]. In other words, the structure subsequently postulated by M. BODANSZKY *et al.*[3] is clearly incorrect.

The mass spectrometric evidence showing A-1437 E to be identical to amphomycin was confirmed by HPLC investigations. In all the systems investigated the antibiotic A-1437 E co-eluted with authentic C_{13}-amphomycin. From this, and from the results described above, it is clear that not only must the composition of the peptide ring be identical in all the lipopeptides investigated, but so too must the configurations of all the amino acids. To provide further confirmation, the peptide A-1437 B was hydrolyzed in constantly boiling hydrochloric acid according to the procedure of BODANSZKY *et al.*[18], and the resulting diaminobutyric acid mixture was preparatively separated as described. NMR spectroscopy revealed the presence of both D-*erythro*-α-β-diaminobutyric acid and L-*threo*-α-β-diaminobutyric acid. The measured optical activity of the latter (+28.7°, *c*1, 6N HCl) corresponds closely with the literature values (+27.1°, *c* 1, in 5N HCl)[19]. The configurations of the other amino acids were investigated by chiral GC analysis. The β-methylaspartic acid present in the acid hydrolysis product is identical with authentic 2*S*,3*S*-β-MeAsp (corresponding to the L-*threo* configuration), as was likewise found by BODANSZKY and MARCONI[20] for the same amino acid in amphomycin. Further GC determinations revealed L-configurations for the aspartic acid, proline, and valine residues, in agreement with the literature data for amphomycin.

NMR Characterization

In most standard solvents, such as d_6-DMSO, d_4-MeOH, or D_2O/H_2O, spectra of poor quality were obtained. The resonances of the amide protons in particular appeared as very broad signals. However, in a 7:1 mixture of acetonitrile and water at 285K (friulimicin B) or a 5.6:1 mixture at 305K (A-1437 A, B, E), well resolved NMR spectra were obtained. Use of various 2D techniques allowed a complete assignment of the proton and carbon chemical shifts for some of the different factors (see Table 3). The data obtained from the HMBC and ROESY spectra confirmed the sequence established by mass spectrometry (Fig. 4). The cyclization between residues 2 and 11 is proved by an intense ROE between Dab^2-βNH and Pro^{11}-Hα.

Further analysis of the ROESY spectrum reveals an intense ROE between the protons at position 2 and position 5, which is only possible with a *cis* arrangement of the double bond in the fatty acid side chain. Selective decoupling of the protons at position 2 allowed the coupling constant between H3 and H4 to be determined. The measured value of 10.7 Hz is also consistent with a *cis* configuration. NMR investigations of the other constituents (A-1437 A, A-1437 B, and A-1437 E) gave the same results.

Discussion

As already listed in the introduction, a number of acidic lipopeptide antibiotics have been reported to date, the majority of which were discovered and investigated before 1970. Like amphomycin too, these are without exception compounds containing a fatty acid residue linked to an aspartic acid *N*-terminal. Of the 8 antibiotics isolable from cultures of the new *Actinoplanes* species HAG 010964, 5 are new compounds. 4 of the 8 substances obtained, including the new compound A-1437 A, belong to the amphomycin group, the remaining 4 differ from the acidic lipopeptides reported in the literature in that the fatty acid is linked to asparagine instead of Asp. Because of their

Table 3. Chemical shifts of friulimicin B and A-1437 B in CD_3CN/H_2O (7 : 1) at 285 K[a].

Residue	Proton/ carbon atom	^1H				^{13}C	
		Friulimicin B	**A-1437 B**	**A-1437 A**	**A-1437 E**	**Friulimicin B**	**A-1437 B**
Asn[1]	NH	7.82	7.71	7.72	7.71	-	-
	α	4.69	4.64	4.63	4.63	53.36	54.54
	β	2.70/2.64	2.58/2.49	2.58/2.49	2.58/2.50	39.42	40.82
	C'	-	-	-	-	174.59	175.64
	β-C'	-	-	-	-	176.23	179.66
	NH_2	7.36/6.76	-	-	-	-	-
Dab[2]	NH	7.77	7.73	7.74	7.74	-	-
	α	5.04	5.07	5.08	5.08	56.49	56.47
	β	4.50	4.49	4.49	4.49	48.88	49.27
	γ	1.05	1.05	1.05	1.05	20.76	20.90
	β-NH	7.59	7.53	7.54	7.53	-	-
	C'	-	-	-	-	172.56	172.88
Pip[3]	α	4.64	4.67	4.67	4.67	57.39	57.64
	β	2.23/1.68	2.26/1.71	2.26/1.71	2.26/1.71	28.86	28.95
	γ	1.64/1.29	1.61/1.32	1.61/1.31	1.62/1.32	22.56	22.60
	δ	1.76/1.70	1.74	1.74	1.72	26.30	26.37
	ε	3.82/3.36	3.81/3.37	3.81/3.36	3.81/3.35	46.41	46.46
	C'	-	-			175.34	175.74
Me-Asp[4]	NH	8.93	8.96	8.95	8.96	-	-
	α	4.56	4.62	4.62	4.62	58.07	58.22
	β	2.51	2.52	2.52	2.53	50.6(broad)	51.02
	γ	1.05	1.07	1.06	1.06	17.24	17.41
	C'	-	-	-	-	176.65	176.69
	β-C'	-	-	-	-	184.13	184.34
Asp[5]	NH	8.24	8.09	8.10	8.09	-	-
	α	4.19	4.22	4.23	4.23	57.09	57.29
	β	2.58/2.50	2.58/2.50	2.59/2.51	2.58/2.52	40.47	42.22
	C'	-	-	-	-	177.34	177.70
	β-C'	-	-	-	-	179.15	179.50
Gly[6]	NH	8.83	8.79	8.79	8.79	-	-
	α	4.23/3.67	4.19/3.69	4.19/3.70	4.19/3.69	45.32	45.61
	C'	-	-	-	-	174.48	174.58
Asp[7]	NH	7.68	7.61	7.61	7.60	-	-
	α	4.57	4.58	4.58	4.58	53.85	53.74
	β	3.18/2.44	3.18/2.45	3.20/2.45	3.19/2.46	41.93	42.10
	C'	-	-	-	-	176.17	176.32

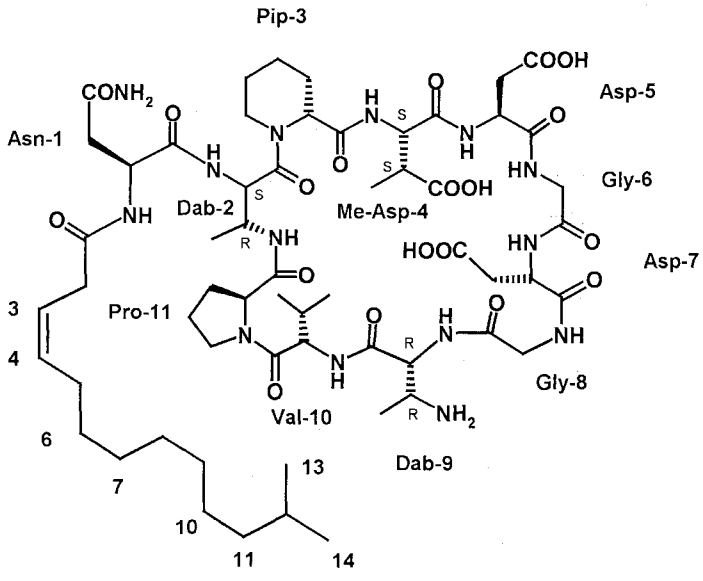
Table 3. Continued

Residue	Proton/ carbon atom	^{1}H				^{13}C	
		Friulimicin B	**A-1437 B**	**A-1437 A**	**A-1437 E**	**Friulimicin B**	**A-1437 B**
	β-C'	-	-	-	-	179.21	179.18
Gly[8]	NH	8.10	7.96	7.98	7.96	-	-
	α	4.29/3.55	4.29/3.58	4.29/3.58	4.30/3.58	45.02	45.28
	C'	-	-	-	-	173.23	173.51
Dab[9]	NH	9.54(b)	9.55(b)	9.52(b)	9.55(b)	-	-
	α	4.43	4.47	4.48	4.48	59.41	59.47
	β	3.54	3.59	3.58	3.59	53.45	53.98
	γ	1.34	1.37	1.36	1.37	19.78	19.83
	C'	-	-	-	-	172.45	172.52
Val[10]	NH	8.31	8.28	8.27	8.28	-	-
	α	4.02	4.03	4.03	4.03	62.58	62.73
	β	2.25	2.24	2.24	2.25	32.20	32.31
	γ	1.05	1.06	1.06	1.07	20.72	20.92
	γ'	0.94	0.95	0.95	0.95	21.39	21.53
	C'	-	-	-	-	175.42	175.51
Pro[11]	α	4.06	4.10	4.10	4.10	63.97	64.12
	β	2.20/1.74	2.21/1.75	2.20/1.75	2.20/1.74	32.50	32.59
	γ	1.99/1.91	2.00/1.91	2.00/1.92	2.01/1.92	27.91	27.99
	δ	3.83/3.55	3.83/3.55	3.83/3.55	3.83/3.55	50.93	51.06
	C'	-	-	-	-	173.57	173.78
FA	1	-	-	-	-	175.52	175.61
	2	3.03	3.02	3.04	3.03	36.84	37.07
	3	5.51	5.51	5.51	5.53	124.41	124.69
	4	5.61	5.60	5.61	5.61	136.65	136.64
	5	2.05	2.06	2.05	2.05	29.82	29.91
	6	1.35	1.35	1.36	1.36	32.40[b)]	32.45[b)]
	7	1.25-1.35	1.25-1.35	1.25-1.35	1.25-1.35	32.14[b)]	32.19[b)]
	8	1.25-1.35	1.25-1.35	1.25-1.35	1.25-1.35	31.97[b)]	32.05[b)]
	9	1.25-1.35	1.25-1.35	1.25-1.35	1.25-1.35	31.83[b)]	31.89[b)]
	10	1.29	1.30	1.30	1.34	29.96	29.98
	11	1.17	1.18	1.18	1.35/1.15	41.54	41.65
	12	1.53	1.54	1.54	0.86	30.51	30.59
	13,14	0.88	0.88	0.88	0.85	24.77	24.86

a) Referenced to sodium-3-(trimethylsilyl)propionate-2,2,3,3-d_4.

b) These signals could not be attributed unambiguously.

Fig. 4. Structure of friulimicin B.

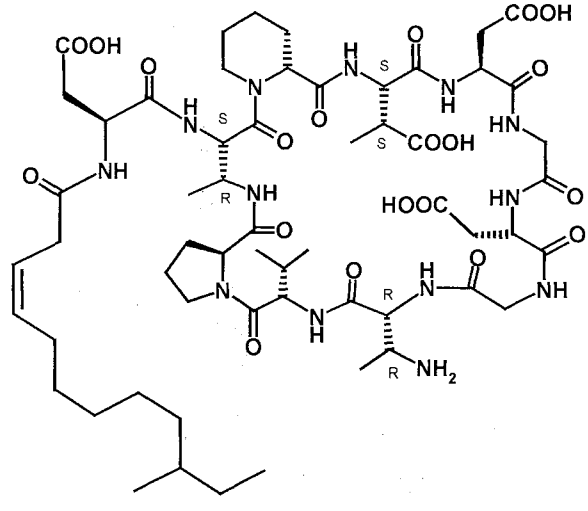


higher isoelectric point, these antibiotics have different physical and biological properties, which allows us to make a comparative assessment of their therapeutic utility.

There are contradictory data in the literature on the structure of amphomycin. Both the nature of the peptide ring and the configuration of the double bond in the fatty acid residue have been formulated differently, as a result of which there are usually also inaccuracies in the review articles. In the work described in this paper the structure of friulimicin B, and hence the structure of amphomycin, have been elucidated by two separate methods, with no discrepancies in the results obtained. Thus, for the principal constituent, Δ3-anteisotridecenoyl amphomycin, we arrived at the structural formula depicted in Fig. 5.

Assuming that the published antibiotics amphomycin[3], tsushimycin, glumamycin, and aspartocin[17] all have the same peptide framework, the only difference being the acyl substituent, then A-1437 B is identical with the principal constituent of tsushimycin, A-1437 G is identical with a constituent of aspartocin, and as already mentioned, A-1437 E is identical with the principal constituent of amphomycin.

Fig. 5. The structure of amphomycin. Only the principal constituent of the amphomycin family, with 3Δ-anteisotridecenoic acid at its *N*-terminal, is depicted.



Experimental

General

Amphomycin was obtained as a gift from Bristol Laboratories, Syracuse, N.Y., USA, and was used without further purification. A second sample was obtained from

Sigma (cat. No. A9835).

The p*Ka* values were measured with a titrator V5.01 from Sirius Analytical Instruments Ltd., Forest Row RH18 5DW, United Kingdom, the average assay temperature was 23.8°C, the average ionic strength 0.159 M. Dynamic light scattering measurements were carried out using a goniometer DLS/SLS-5000 from ALV GmbH, D-63225 Langen, Germany, with green polarized Nd-YAG laser light, 532 nm. The apparatus was equipped with a single mode faser and a SO-SIPT photo multiplier from ALV GmbH. The angle of detection was 90°, the measurements were done at 22°C. The following mass spectrometric techniques were performed using a MAT 95Q instrument (Finnigan): electron impact (EI, 70 eV), chemical ionization (isobutane), high-resolution EI and CI measurements (peak matching), and fast atom bombardment (FAB; matrix: 3-nitrobenzyl alcohol). Electrospray ionization (ESI) measurements were conducted on TSQ 700 (Finnigan) and LCQ (Finnigan) instruments.

Analytical Chromatography

The concentrations of A 1437 lipopeptides were determined according to a reversed-phase HPLC method using the following equipment: autosampler (TSP, model SP 8880), low pressure HPLC pump (LKB, type 2150), photometric detector (Jasco UV-975, 210 nm wavelength), and a TSP Chromjet integrator. The separations were performed using a steel column (4.0 mm×250 mm) packed with Lichrospher 60 RP-select B, 5 μm (E. Merck, Darmstadt, Germany) as the stationary phase. The mobile phase was acetonitrile/water/85% phosphoric acid (450 : 550 : 1). The analyses were carried out at a flow rate of 1.0 ml/minute and an injection volume of 20 μl.

Isolation

180 liters of the culture filtrate of *Actinoplanes friuliensis* HAG 010964, DSM 7358, fermented in an L-valine-supplemented complex nutrient medium described by ARETZ[15] *et al.*, was loaded onto a column packed with 17 liters of Diaion HP 20 adsorption resin (Mitsubishi Chemical Corporation, Tokyo 100, Japan). The column was washed with 25 liters water and 30 liters of 10% 2-propanol in 10 mM pH 7.2 sodium phosphate buffer, and was then eluted with 50% 2-propanol in 10 mM pH 7.2 sodium phosphate buffer. The elution fractions were monitored by HPLC and the lipopeptide-containing fractions were pooled. The combined eluate, which contained about 30 g of the antibiotic mixture, was concentrated by ultrafiltration using a LAB module 20 (De Danske Sukkerfabrikker, DK 4900, Nakskov) and UFCA 1 membranes supplied by Hoechst AG Frankfurt/M. The concentrated, desalted solution was loaded onto a 3.5 liters DEAE Sepharose fast-flow (Pharmacia Fine Chemicals, Uppsala, Sweden) column equilibrated to pH 6.8 with phosphate buffer. The anion exchange resin was then eluted with a linear gradient of 10 mM sodium phosphate (pH 6.8) buffer/methanol (1 : 1) to 80 mM sodium phosphate (pH 6.8) buffer/methanol (1 : 1). At 25~33 mM phosphate buffer friulimicin A and B were mainly eluted, and at 40~55 mM buffer the acidic constituents A-1437 A and B. After HPLC analysis the fractions respectively containing friulimicin A and B and A-1437 A and B were combined, concentrated by ultrafiltration, and separated on a 2 liter column packed with CHP20P MCI gel, 75~150 μm (Mitsubishi Chemical Corporation, Tokyo 100, Japan). This column was eluted with a linear gradient of 20 mM sodium phosphate buffer (pH 7.2) (buffer A) to 30% acetonitrile in 20 mM sodium phosphate buffer (pH 7.2) (buffer B). In the case of the friulimicin mixture, the first compound washed from the column was friulimicin A, followed by friulimicin C, then the principal constituent friulimicin B, and finally traces of D. The fractions containing pure compounds were desalted on CHP20P MCI gel, 75~150 μm, eluting with a gradient of water to 50% propanol in water. Concentration of the appropriate fractions under reduced pressure, followed by freeze-drying gave 154 mg of friulimicin A and 3.1 g of friulimicin B. Analogous work-up of the later fractions from the ion-exchange column afforded 145 mg of A-1437 A and 5.2 g of A-1437 B.

48 liters of culture filtrate of *Actinoplanes friuliensis* HAG 010964, fermented in an L-isoleucine-supplemented nutrient medium as described above, was isolated by solid-phase extraction, separated on a 1 liter DEAE Sepharose fast flow column, and concentrated by ultrafiltration. The individual constituents were separated on an ODS column, (LiChrosorb RP-18, 10 μm, 5×25 cm), with an elution gradient of 5~35% acetonitrile in 10 mM sodium phosphate buffer. The separated compounds were then desalted to give 1 g A-1437 E, 1.4 g friulimicin C, 1.1 g A-1437 G, and 1.4 g friulimicin D.

NMR Spectroscopy

All NMR spectra were recorded on Bruker DRX 600 spectrometers operating at 600 MHz. All spectra of friulimicin B were acquired at 7°C using a solution of 5 mg of peptide in 0.6 ml CD_3CN/H_2O (7 : 1). All spectra of A1437-A, A1437-B, and A1437-E were acquired at 32°C using a solution of 5 mg of peptide in 0.6 ml CD_3CN/H_2O

(5.6 : 1). The data were processed on an indigo2 station (Silicon Graphics) using Bruker XWINNMR software.

Homonuclear COSY[21], TOCSY[22], and ROESY[23] experiments were performed with a sweep width of 10 ppm. In all experiments, spectra were recorded with 512 increments in t_1 and 4096 complex data points in t_2. For the ROESY spectra, 32 transients were averaged for each t_1 value, and for COSY and TOCSY, 8 transients. The TOCSY (spin-lock field 10 kHz, mixing sequence MLEV17[22]) and ROESY spectra were recorded using mixing times of 80 or 250 mseconds respectively. Solvent suppression was achieved by continuous irradiation during the recycle delay.

For the HMQC spectra[24], 512 increments (16 scans) with 2048 complex data points in t_2 were collected using a sweep width of 10 ppm in the proton, and 165 ppm in the carbon dimension. The HMBC spectra[25] were acquired with a sweep width of 10 ppm in the proton, and 200 ppm in the carbon dimension. A total of 48 transients were averaged for each of 512 increments in t_1, and 2048 complex points in t_2 were recorded. A delay of 70 mseconds was used in the development of long range correlations.

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